A STUDY OF BACTERIAL AND FUNGAL PATHOGENS WITH EMPHASIS ON MYCOPLASMA IN LOWER RESPIRATORY INFECTIONS IN HIV SERO-POSITIVE PATIENTS

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DECLARATION

I, Dr. S. Nirmala solemnly declare that the dissertation titled ***"A STUDY OF BACTERIAL AND FUNGAL PATHOGENS WITH EMPHASIS ON MYCOPLASMA IN LOWER RESPIRATORY INFECTIONS IN HIV SERO-POSITIVE PATIENTS"*** *w*as done by me at Coimbatore Medical College Hospital during the period from January 2005 – August 2006 under the guidance and supervision of Dr. R.K. Geetha, M.D., D.C.P., Professor and Head of the Deparment of Microbiology.

This dissertation is submitted to the Tamilnadu Dr. M.G.R. Medical University towards the partial fulfillment of the requirement for the award of M.D. Degree (Branch – IV) in Microbiology.

I have not submitted this dissertation on any previous occasion to any University for the award of any degree.

Place:

Date: **Dr. S. NIRMALA**

CERTIFICATE

This is to certify that the enclosed work **"*A STUDY OF BACTERIAL AND FUNGAL PATHOGENS WITH EMPHASIS ON MYCOPLASMA IN LOWER RESPIRATORY INFECTIONS IN HIV SERO-POSITIVE PATIENTS*"** submitted by Dr. S.Nirmala to the Tamilnadu Dr. M.G.R. Medical University is based on bonafide cases studied and analysed by the candidate at the Department of Micobiology, Coimbatore Medical College Hospital during the period from January 2005-August 2006 under my guidance and supervision and the conclusions reached in this study are her own.

Dean

Coimbatore Medical College

Signature of the Guide

(Professor & Head Of the Department Of Microbiology)

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LIST OF ABBREVIATIONS

1.	HIV	Human Immuno Deficiency Virus
2.	LRTI	Lower Respiratory Tract Infection
3.	AIDS	Acquired Immuno Deficiency Syndrome
4.	Mp	Mycoplasma pnemoniae
5.	SDA	Sabouraud's Dextrose Agar
6.	LJ	Lowenstein Jensen's
7.	CLSI	Clinical laboratory Standards Institute
8.	TBO	Toluidine blue O
9	AFB	Acid fast bacilli
10.	PPLO	Pleuropneumonia like organisms
11.	SP_4	Soy Peptone
12.	ELISA	Enzyme linked immunosorbent assay
13.	PCR	Polyamerase Chain Reaction
14.	RNA	Ribonucleic acid
15.	DNA	Deoxyribonucleic acid
16.	ISR	Immune Status Ratio
17.	IS	Immuno suppression

LIST OF COLOUR PLATES

INTRODUCTION

Human immunodeficiency virus (HIV) infection / Acquired immune deficiency syndrome (AIDS) is a global pandemic [1] and is the most important public health problem of the 21 st century[2,3]. Though HIV made a delayed entry into India in 1986 its spread has been very rapid and at present is in an advanced stage of the pandemic in some states of our country [4,5].

India has a population of one billion with HIV infection spreading among them at an increasing rate. The spread of HIV in India has been diverse with much of India having a low rate of infection and the epidemic being most extreme in the southern half of the country and in the far north-east. The highest HIV prevalence rates are found in Maharashtra, Andhra Pradesh, Karnataka, Nagaland, Gujarat, Tamil Nadu, Manipur and West Bengal.

The Indian National AIDS control organization (NACO) estimates that 5.21 million people were living with HIV in 2005 giving an adult prevalence of 0.91%. It also estimates that 111,608 people were living with AIDS at the end of July 2005 [6] and the average HIV prevalence among women attending antenatal clinics in India is 0.88% and in Tamil Nadu is 0.5%[7].

HIV is unique among human viral infections. Once infection has established, the virus succeeds in escaping immune mediated clearance and is virtually never eliminated from the body. A chronic infection develops that persists with varying degrees of viral replication for a longer period along with progressive qualitative and quantitative deficiency of the subset of T-lymphocytes which leads to gradual loss of immune system functions and predisposes to several opportunistic

infections particularly respiratory infections due to various bacterial and fungal pathogens[8] which account for 70% of AIDS defining illness[9-11]

Lower respiratory tract infections are an important cause of morbidity and mortality in all age groups in HIV patients[12]. In India it has been reported that respiratory infections account for 20 – 40% of outpatient and 12 – 35% of inpatient attendance in a general hospital in HIV patients[13,14]. Since the etiologic agents of lower respiratory tract infections cannot be determined clinically microbiological investigations are critical for both treatment and epidemiological purposes as lower respiratory tract infections form an important part of opportunistic infections in HIV infected persons[15-17].

In our hospital we have also noticed an increasing incidence of lower respiratory tract infections in HIV infected patients. This led us to conduct a prospective study in order to record clinical and microbiological observations and to find out the bacterial and fungal etiology of lower respiratory tract infections in HIV seropositive patients.

We also did this study to find the incidence of mycoplasma infection in lower respiratory tract infections in HIV seropositive patients as mycoplasma species act as important cofactors in HIV infection.

AIMS AND OBJECTIVES

1. To evaluate the bacterial and fungal etiology of lower respiratory tract infections in the HIV sero-positive patients.
2. To study the incidence of Mycoplasma pneumoniae among the bacterial isolates
3. To ascertain the significance of serology in diagnosis of Mycoplasma pneumoniae infection in HIV sero-positive patients.
4. To study the various haematological and clinico-radiological findings in lower respiratory tract infections in HIV sero-positive patients.
5. To assess the antibiotic susceptibility pattern of various bacterial isolates in HIV sero-positive patients.

REVIEW OF LITERATURE

Pulmonary disease is the first clinical presentation and most frequent complication of HIV infection and the most common manifestation of pulmonary disease is the lower respiratory tract infection particularly pneumonia of bacterial etiology. The incidence and severity of lower respiratory tract infections(LRTI) increase with degree of immunosuppression[1]. At the acquired immuno deficiency syndrome stage the responsible bacteria and clinical presentation may be atypical[18,19]. Bacterial and fungal pneumonias may be fatal particularly in AIDS and its occurrence is predictive of a reduced survival time.

HIV associated respiratory infections include upper respiratory tract infections i.e. acute or chronic sinusitis, pharyngitis and lower respiratory tract infections – acute or chronic bronchitis, pneumonias[1]. These are prevalent during all stages of HIV infection. Many cases of LRTI in HIV patients present with high frequency of bacteremia, unusual radiographic abnormalities, high rate of pleural effusions and bacterial pneumonias due to opportunistic bacteria and hence these cases have to be studied.[20]

Risk factors for LRTI

The most important risk factor is the degree of immunosuppression as reflected by the CD4 + T lymphocyte count. Acute bronchitis is highly prevalent during all stages of HIV disease whereas pneumonias were clearly related to decreased CD4 count.[21,22] A similar relationship between a decreased CD4 count and an increased risk of LRTI has also been found in European studies.[23,24]

Hirschtick et al [25]showed that the risk was greater in intra venous drug users than in homosexual male or female partners. In the same study tobacco was equally shown to be an independent risk factor in the subgroup of HIV sero-positive subjects with < 200 CD4 lymphocytes/mm^3.

Another risk factor is neutropenia which may result from direct retroviral infection, the use of antiretroviral and other drug therapy, systemic opportunistic infections and autoimmune mechanisms. [26]Splenectomy,[27,28] previous pneumonia whatever the cause [29] and smoking illicit drugs have also been associated with bacterial pneumonia.

Regarding specifically nosocomial pneumonia, advanced HIV infection, CNS diseases have been shown to be risk factors in the case control study of Tumbarello et al[30].

Bacterial and fungal etiology of LRTI

The following pyogenic bacteria and fungi have been recognized to be a major cause of lower respiratory tract infection[10,11] in HIV infected patients whatever their level of immunosuppression [1].

TABLE 1 - Bacterial and fungal etiology of LRTI

Infection	Common	Less common
Bacteria	Streptococcus pneumoniae Haemophillus influenzae Staphylococcus aureus Pseudomonas aeruginosa	Moraxella Legionella Rhodococcus equi Nocardia
Mycobacteria	Mycobacterium tuberculosis	M. avium intracellulare M. kansasii M. Xenopi
Mycoplasma	Mycoplasma pneumoniae	Mycoplasma fermentans Mycoplasma pirum Mycoplasma penetrans Mycoplasma genitalium
Fungi	Pneumocystis carini Histoplasma capsulatum Cryptococcus neoformans Aspergillus species	Candida albicans Coccidioides immitis Penicillium marneffei Blastomyces dermatitidis

Incidence of bacterial Lower respiratory tract infection

The true incidence of LRTI in HIV infected patients is difficult to assess and varies with the population surveyed[9]. Acute bronchitis and bacterial and fungal pneumonias are the most common respiratory diseases of HIV infected patients. The respective incidence per 100 person years were 13.7 for bronchitis and 5.5 for pneumonias. When HIV infected individuals were compared with controls of similar age race, sex and transmission group, the incidence rates were two fold greater for bronchitis and six fold greater for pneumonias[21].

In intra venous drug users the incidence of pneumonia per 100 person years was 1.93 in HIV sero-positive and 0.45 in HIV sero-negative subjects[29]. Boschini et al[23] reported 149 episodes of community acquired pneumonia(CAP) among HIV sero-positive patients and 61 among HIV sero-negative subjects with incidence rates per 1,000 person years of 90.5 and 14.2 respectively. The incidence rates of pneumonia per 1,000 person years were 38.6 in HIV sero-positive females and 3.7 in HIV sero-negative females in Nairobi, Kenya among female sex workers. More precisely, the incidence rates of invasive pneumococcal disease and pneumococcal bacteraemia per 1,000 person years were 42.5 and 23.8 in HIV sero-positeve females and 3.7 and 0 in HIV sero-negative females respectively[18]. The relative risk of development of invasive pneumococcal disease with underlying HIV infection was 17.8 (95% confidence interval 2.5 – 126.5). Pyogenic bacteria as well as Mycobacterium tuberculosis were recognized as major cause of respiratory diseases in HIV infected Africans[30-32].

The first series investigating bacterial pneumonia in patients with AIDS or ARC have shown the predominant role of Streptococcus pneumoniae and to a lesser degree, Haemophilus influenzae, in adults as well as children[33] in developing countries [32-34] and developed contries[35-38]. Subsequently other series with greater number of patients followed up over a long period have emphasized that other bacteria have also been the cause in HIV infected patients.

Typical pyogenic bacteria more particularly S. pneumoniae and H. influenzae were the major responsible bacteria. In a French clinical epidemiology database,

S. pneumoniae and H. influenzae were the cause of 52 and 16% of bacteriologically confirmed pneumonia respectively[39]. Hirschtick et al [25],found S. pneumoniae and H. influenzae in 52 and 15% of patients with confirmed pneumonia respectively. In the African cohort study by Gilks *et al.*[34], 91% of pneumococcal serotypes incriminated in HIV-seropositive adults were 1, 3, 5, 7, 19 and 23.

Klebsiella pneumoniae, other members of Enterobacteriaceae family and Pseudomonas aeruginosa were present in 13,10 and 8% of cases respectively of confirmed pneumonia in a study by Hirschtick et al[25]. Pseudomonas aeruginosa and S.aureus were cause of 25,9% of CAP in a study by Afessa et al[40]. Levin et al[41] recovered S.aureus in 23% of sputum cultures in 129 HIV infected patients.Nocardia species[42],Rhodococcus equi[43],Streptomyces sp[44] were found to be responsible for chronic pneumonia in AIDS. Pasteurella multocida[45],Bordetella bronchiseptica[46],Neisseria sp[47],Rocholimea sp[48],Corynebacterium pseudodiphtheriticum[49],Legionella[50] were equally the cause of pneumonia in HIV patients.Casedo et al[51] showed lung involvement in AIDS patient with Salmonella septicaemia.

Tuberculosis (TB) ranks as the most common infection seen in the developing countries. About 55 to 89% of AIDS patients in India were found to be suffering from extensive pulmonary tuberculosis[52,53]. It is estimated that world wide, nearly 2 billion people are infected with Mycobacterium tuberculosis, 42 million are HIV infected and 5 to 6 million are dually infected with M. tuberculosis and HIV. 70% of HIV / TB dually infected people live in Sub-saharan Africa and 20% in Asia[11].

Incidence of Fungal lower respiratory infection

Pneumocystis jiroveci previously known as Pneumocystis carinii is the most common opportunistic infection in the West (>60%) and is found in patients with profound immunosuppression (CD 4 count < 200 cells/mm3). Unlike in the west, the incidence of PCP is negligible in India (about 12%)[11]. This is possibly due to poor index of suspicion and extensive use of cotrimoxazole for prophylaxis of PCP in HIV[54].

Cryptococcus neoformans, an encapsulated yeast is the most common deep seated fungal infection in HIV patients. Though the cryptococcal meningitis is the commonest presentation, lungs is the primary site of infection. In a study from Rwanda, Cryptococcus neoformans was isolated from sputum specimen of 37 HIV infected patients[55].

Meyohas in his study on AIDS patients has isolated Aspergillus species from patients on anti-retroviral and steroid therapy[56]. Shivananda in his study in 1992 first reported 15.3% of isolates to be Aspergillus species[57]. Geetha Lakshmi from Chennai has documented pulmonary aspergillosis in 36 HIV infected patients[58].

Though pulmonary candidiasis is documented to be a very rare disease occurring in late stages of AIDS, oral and esophageal candidiasis is reported as second most common (58%) of opportunistic infection among HIV patients from India[54].

Pencillium marneffei, a dimorphic fungus has been reported most commonly from South east Asian countries in HIV patients[10]. Endemic mycosis such as

Histoplasmosis, Coccidioidomycosis, Blastomycosis are not seen very frequently in India[11].

Incidence of mycoplasma in HIV

Mycoplasmas are commensals causing self-limiting and clinically unimportant infections in human beings. Recent isolation of these organisms from adults with AIDS suggests that mycoplasmas might act as cofactors in patients infected with human immunodeficiency virus (HIV)[59].

The five species of mycoplasmas identified as being associated withAIDS include *Mycoplasma pneumoniae,M. fermentans*, *M. pirum*, *M. penetrans* and *M. genitalium*[60-61].

Teel *et al* [65] have reported that mycoplasmas colonized the respiratory tracts of 28 per cent of HIV-positive and 10.5 per cent of HIVnegative patients.

A detection rate of 12.5 per cent of mycoplasmas has been documented from bronchoalveolar lavage specimens of HIV infected patients which indicated that AIDS patients might be more often colonized or infected by mycoplasmas than HIV-negative patients or other immunocompromised persons [64].

Hjordis [62] isolated *M. pneumoniae* from respiratory specimens from AIDS patients by culture. Ainsworth *et al* [63] have detected the presence of *M. fermentans* in the respiratory tract of 27 per cent of the HIV population.

In another study mycoplasmas have been shown to colonize HIVpositive patients to a larger extent than HIV-negative individuals[62].

Mycoplasma as cofactor in hiv progression[66-71]

Luc Montagnier,co-discoverer of AIDS virus proposed that HIV may not work alone and mycoplasmas could be playing the key role in HIV progression[68]. Pathogenic *Mycoplasma* species may influence HIV pathogenesis by specific and direct activation or suppression of the immune system, the production of superantigens with subsequent alterations in immune responses, or their contribution to the oxidative stress observed in HIV-positive patients. Also, the development of AIDS may increase the susceptibility of HIV-infected patients for coinfection with various *Mycoplasma* species able to bind HIV capsid protein gp120 permitting adhesion of HIV virions to the mycoplasma surface. Subsequently the HIV viruses could be transported directly to cells expressing CD4 receptors. After binding to target cells, mycoplasmas can stimulate host cell activation by IL-1 and TNFa, which are known effectors for virus reproduction. In addition, oligosaccharides of the mycoplasmal glycocalyx may protect bound HIV-1 virons from host immune responses. Antigen similarities between the surface components of mycoplasmas and HIV-1 have led to speculation that they use similar mechanisms for cell entry. For example, the HIV1 gp120 envelope glycoprotein and *Mycoplasma* adhesion proteins share sequence homology and also have significant similarity with the CD4-binding site of the class II major histocompatibility complex (MHC) proteins. The interactions of microorganisms with MHC-related antigens on host cells could contribute to a number of possible outcomes, including T-cell dysfunction, T-cell depletion, T-cell shift, B-cell proliferation, hyperglobulinemia and antigen-presenting cell dysfunction.

Historical background

- The first mycoplasma to be isolated in culture was the bovine pleuropneumonia agent now known as *Mycoplasma mycoides* subsp. *mycoides*, which was described initially by Nocard andRoux in 1898 [72]
- In the 1930s Klieneberger introduced the concept that mycoplasmas were "L-forms" of bacteria lacking cell walls and living symbiotically with other, walled bacteria [73].
- Dienes and Edsall detected the first mycoplasma isolated from humans in a Bartholin's gland abscess in 1937 [74]. This mycoplasma was probably the organism we now know as *Mycoplasma hominis*.
- The organism eventually known to be *Mycoplasma pneumoniae* was first isolated in tissue culture from the sputum of a patient with primary atypical pneumonia by Eaton et al in 1944, and thereafter it became known as the Eaton agent [75].
- In 1961 Marmion and Goodburn postulated that the Eaton agent was a PPLO and not a virus[76,77].
- Chanock et al. succeeded in culturing the Eaton agent on cell-free medium and proposed the taxonomic designation *M. pneumoniae* in 1963.

Mollicute taxonomy and classification

The term “mycoplasma” (Greek; “mykes” - fungus and“plasma” - formed) emerged in the 1950s [78] and replaced the older PPLO terminology. In the 1960s, mycoplasmas were designated members of a class named *Mollicutes*,which derives from Latin words meaning soft (“mollis”) and skin (“cutis”). Members of the class *Mollicutes* are characterized by their small genomes consisting of a single circular chromosome containing 0.58 to 2.2 Mbp, a low G+C content (23 to 40 mol%), and the permanent lack of a cell wall [80].

M. pneumoniae is a member of the family *Mycoplasmataceae* and order *Mycoplasmatales*. Studies of 16S rRNA sequences suggest that mycoplasmas are most closely related to the gram-positive eubacterial subgroup that includes the bacilli, streptococci, and lactobacilli[79].

Cell biology

Mycoplasmas represent the smallest self-replicating organisms, in both cellular dimensions and genome size, that are capable of cell-free existence[81]. Individual spindle-shaped cells of *M. pneumoniae* are 1 to 2 μm long and 0.1 to 0.2 μm wide, compared with a typical bacillus of 1 to 4 μm in length and 0.5 to 1.0 μm in width. Accordingly, the *M. pneumoniae* cell volume is less than 5% of that of a typical bacillus. Cells may either divide by binary fission or first elongate to multinucleate filaments, which subsequently breakup to coccoid bodies

The small size and volume of mycoplasmal cells allow them to pass through 0.45μm-pore-size filters that are commonly used to filter sterilize media. The small cellular mass also means that mycoplasmas cannot be detected by light

microscopy, and they do not produce visible turbidity in liquid growth media. Typical colonies of *M. pneumoniae*, show fried egg appearance, rarely exceed 100µm in diameter when cultivated on enriched medium and require examination under a stereomicroscope to visualize their morphological features. The genome of *M. pneumoniae* was completely sequenced in 1996 and shown to consist of 816,394 bp with 687 genes. Mollicutes have no ability to synthesize peptidoglycan cell walls, since the genes responsible for these processes are not present in the genome. The lack of a rigid cell wall confers pleomorphism on the cells and makes them unable to be classified as cocci or bacilli in the manner of conventional eubacteria. Sterols are necessary components of the triple-layered mycoplasmal cell membrane that provide some structural support to the osmotically fragile mycoplasma.Maintenance of osmotic stability is especially important in mollicutes due to the lack of a rigid cell wall. Another structural component of the *M.pneumoniae* cell that is important for extracellular survival is aprotein network that provides a cytoskeleton to support thecell membrane

M. pneumoniae has been shown to bind on glass and other solid surfaces, with the organism moving with the attachment organelle at the leading end. Neithergenomic analysis nor electron microscopy of *M. pneumoniae*

has demonstrated the presence of structures such asflagella or pili, suggesting that gliding motility occurs by an unknown mechanism involving the attachment organelle[82].

Pathogenesis

M.*pneumoniae* is primarily an extracellular pathogen that depends on close association with host cells to survive, it has evolved a complex and specialized attachment organelle to facilitate its parasitic existence .This attachment organelle consists of a specialized tip structure with a central core of a dense rodlike central filament surrounded by a lucent space that is enveloped by an extension of the organism's cell membrane[83,84]. The tip structure is actually a network of adhesins, interactive proteins, and adherence accessory proteins that cooperate structurally and functionally to mobilize and concentrate adhesions at the tip of the organism.

The P1 adhesin[85,86] is a 170-kDa protein concentrated in the attachment tip that is now known to be the major structure responsible for interaction of *M. pneumoniae* with host cells. P30 [87] is one of several additional proteins that have been implicated in the adherence process, based on the knowledge that antibodies developed against P30 can block *M. pneumoniae* hemadsorption Other structures produced by *M. pneumoniae* that have been studied as mediators in cytadherence in *M. pneumoniae* include proteins [88] HMW1, HMW2, HMW3, HMW4, HMW5, P90, and P65, which, in addition to P30, are believed to participate in the establishment of the polar structure. Once this polar structure is established, an independently assembled complex of proteins B, C, and P1 is drawn to the structure to complete formation of the functional terminal attachment organelle .

Hydrogen peroxide and superoxide radicals are synthesized as a result of a flavin-terminated electron transport chain *M.pneumoniae* act in concert with endogenous toxic oxygen molecules generated by host cells to induce oxidative stress in the respiratory epithelium[89]. Once *M. pneumoniae* reaches the lower respiratory tract, the organism may be opsonized by complement or antibodies macrophages become activated, begin phagocytosis, and undergo chemotactic migration to the site of infection[90]. High percentages of neutrophils and lymphocytes are present in alveolar fluid. Lymphocyte proliferation, production of immunoglobulins, and release of tumor necrosis factoralpha (TNF-α), gamma interferon (IFN-gamma), and various interleukins(including interleukin-1 [IL-1], IL-2, IL-4, IL-5,IL-6, IL-8, IL-10, and IL-18) occurs[91]

Immune Response and Immunomodulatory Effects[82]

Following an initial infection, the normal immune system responds by rapidly producing antibodies that peak after 3 to 6 weeks, followed by a gradual decline over months to years.

Elevation of M. pneumoniae-specific IgM alone can often be interpreted as evidence of acute infection, since this antibody typically appears within 1 week of the initial infection and approximately 2 weeks before IgG antibody.

In these cases, reinfection leads directly to an IgG response; therefore, the absence of a positive IgM test does rule out an acute infection. When it does occur, the IgM response may persist for months or years following infection and in these cases a positive IgM test result may not reflect a current or recent infection.

IgA, while often overlooked as a diagnostic antibody class, may actually be a better indicator of recent infections in all age groups. IgA antibodies are produced early in the course of disease, rise quickly to peak levels, and decrease earlier than IgM or IgG.

In addition to M. pneumoniae-specific antibodies, a variety of cross-reactive antibodies may develop in association with M. pneumoniae infection. The extensive sequence homology of the M. pneumoniae adhesin proteins and glycolipids of the cell membrane with mammalian tissues is a well-known example of molecular mimicry that may trigger autoimmune disorders that involve multiple organ systems through formation of antibodies against substances such as myosin, keratin, fibrinogen, brain, liver, kidney, smooth muscle, and lung tissues.

Antigenic Variation

High-frequency phase and antigenic variation of surface adhesin proteins made possible by DNA rearrangements in truncated and sequence-related copies of the P1 adhesin genes that are dispersed throughout the genome has been described for M. pneumoniae[82]. Recombinational events among the repetitive elements themselves and with regions of the three-gene P1 adhesin operon promote diversity and altered specificities and affinities and maximize the coding potential of the limited mycoplasma genome.

Clinical significance

M.pneumoniae is one of the many causes of a pneumonic process called atypical pneumonia along with several bacterial (Chlamydia pneumoniae, Legionella pneumophila) and fungal (Pneumocystis carinii) pathogens. About 10-30% of CAP are caused by M.pneumoniae infection and these cases represent only 3-10% of M.pneumoniae infection since tracheobronchitis or URT develop in most individuals or remain asymptomatic. Infection usually occurs in school age children >5 yrs, adolescents, young adults in normal population. Most infection in these are minor and include pharyngitis, tracheobronchitis, bronchiolitis and croup. Severe infection develops in immuno compromised HIV patients and elderly requiring hospitalization and death may occur in some[92].

Incubation period is 2-3 weeks. Clinical presentation is usually insidious with the gradual onset of constitutional and pneumonic symptoms. Fever of 101-102^{0}F with chills, malaise, head ache, sore throat, nasal congestion, dry non productive cough appear early in the course. The leucocyte count is often normal or elevated in immuno compromised patients. LRT symptoms develop early and sputum becomes more mucoid or mucopurulent and hemoptysis may also occur. Gram stain smear of sputum show few to moderate amounts of PMN infective cells and no organisms. With the onset of pneumonic symptoms patients may feel as if they have severe cold and flu, yet will continue to function normally hence the application of the term walking pneumonia to this disease. On the chest examination, localized bronchi and scattered rales are usually detected and findings on chest radiograph are consistent with diffuse

bronchopneumonia generally involving multiple lobes of the lung without consolidation.X ray pattern may vary widely and may show peribronchial infiltrates, atelectasis and hilar lymphadenopathy. Radiologic findings reveal more than the physical examinations of patients. Other pleuro-pulmonary complications like lung abscess and pneumothorax are also uncommon. Abnormalities on chest films resolve more slowly in 10 days to 6 weeks. Recurrence and relapses of pneumonia despite appropriate antimicrobial therapy can also occur in HIV patients.

Complications

Dermatologic, cardiovascular, musculoskeletal, neurologic, urologic, hepatobiliary or ocular complications can occur. Humoral and cellular Immunodeficiency states in HIV patients predispose than to serious disease with M pneumonia.These patients may suffer repeated bouts of M pneumonia and have difficulty in eliminating the organism from respiratory tract despite adequate therapy.These patients often have upper and lower respiratory tract symptoms with few or no infiltration observed on chest X-ray and have significant complications. Fulminant dessiminated infection with multi system involvement is rare. But it has been reported.

Media for culture of Mycoplasmas:

Human mycoplasmas differ in their optimal pH for growth and in the atmospheric conditions that are required for successful recovery from clinical specimens. Media for isolation of M.pneumoniae are buffered at an optimum pH

of about 7.8. The optimum temperature for mycoplasmal growth is 35-37^{0}C. M.pneumoniae grows well in air or in an atmosphere of 95% N and 5% CO_2

Several types of media have been described for the cultivation of M. pneumoniae.The medium recommended by Center for disease control for isolation of M. pneumoniae is Methylene blue glucose diphasic medium also known as PPLO diphasic medium. This medium contains PPLO (Pleuropneumonia like organism) broth and agar, yeast extract, serum supplements along with glucose, Methylene blue and phenol red. The methylene blue in the medium inhibits the growth of other human mycoplasmas that may be found in respiratory tract making the medium selective for M. pneumoniae. During growth of M. pneumoniae the medium becomes more acidic and the phenol red turns colour from salmon to yellow. At the same time the organisms reduce the methylene blue and turn it from blue to colourless. Therefore the colour of the broth phase changes from purple to green or yellow green while the agar phase turns from purple colour to yellow or yellow orange. This medium is used in conjunction with mycoplasma glucose agar(PPLO agar) medium. Colonies recovered either directly on this medium or from subcultures of positive broth organ subjected to inspection and identification procedures. Inorder to avoid overgrowth by other pathogens a broad spectrum beta lactum antibiotic and an antifungal agent should be added to the medium.

Another medium that is recommended for isolation for Mycoplasma pneumoniae from clinical specimens is Soy peptone (SP_4) Broth and SP_4 agar containing glucose.

Isolation and identification

The growth of M. pneumoniae from clinical specimens is detected by the ability of these organisms to produce acid from glucose. Methylene blue glucose diphasic medium is inoculated with 0.2 ml of specimens and broth cultures are incubated at 35^0C with caps tightened. Tubes are inspected daily for colour changes and turbidity for 4 weeks. A slight, gradual shift in the pH indicator over 8 to 15 days without gross turbidity suggests true positive cultures. As soon as the colour changes are apparent, the broth is sub-cultured onto agar medium and incubated for 5 to 7 days at 35^0C. Agar plates are sealed with air permeable cellophane tapes to prevent agar from drying out. Inspection of agar under low power of microscope will reveal fried egg colonies. In the absence of obvious colour change a blind subculture to agar media should be performed after 1 and 3 weeks of incubation. A general scheme for isolation of M. pneumoniae is shown in chart.

Human Mycoplasmas can be divided into three groups on the basis of utilization of three substrates – glucose, arginine and urea. An enriched peptone basal medium containing yeast extract and serum supplemented with one of the three substrates with a pH indicator is used. Mycoplasma pneumoniae metobolises glucose to produce lactic acid resulting in a shift to acidic pH.

Supravital dyes such as Diene's stain can be used for identification and characterization of the colonies. Tests such as Haemadsorption, tetrazolium reduction, serological methods like epifluorescence procedure, growth inhibition test, immunoblot technique are also used for culture confirmation.

Serology

Serology is an important tool for the diagnosis of M. pneumoniae infection. This is due to the ease of specimen collection and wide spread availability of serological test[82]. Before the availability of more advanced serologic techniques, detection of cold agglutinins was a valuable tool for M. pneumoniae diagnosis[96]. The formation of cold agglutinins is the first humoral response to M. pneumoniae. However cold agglutinins are not reliable indicators as they are elevated in only 50 to 60 % of patients. Cross reactions may be induced by Ebstein-Barr virus, Cytomegalo virus and Klebsiella pneumoniae, malignancies of lymphoid cells and auto-immune diseases[93]. Complement fixation test(CF) once considered as standard for diagnosing M. pneumoniae is not widely used due to lack of both sensitivity and specificity. More advanced alternative to CF text is the microparticle agglutination assay (MAG), serodiamyco II. The principle of this test is Haemagglutination by specific antibodies to mycoplasma pneumoniae. Erythrocytes are replaced by latex particles to avoid non-specific reactions. Neither the CF test nor MAG enable differentiation between the antibody classes. So in order to achieve a specific diagnosis, separate detection of IgM, IgG, IgA antibodies based on ELISA principle is helpful. A rapid card based assay (immunocard mycoplasma) has also been developed for IgM determination. Western immunoblot technique for M. pneumoniae has also been recently developed. Antigen detection techniques include, direct immuno-fluorescence, counter-immuno-electrophoresis, immuno-blotting, antigen capture enzyme immuno-assay[97]

Molecular techniques :

Hybridisation assays provide a similar sensitivity as ELISA tests for antigen detection. The Genprobe rapid system which involves a I^{125} labelled DNA probe to an rRNA sequence specific for M.pneumoniae was widely used before the application of PCR[82,96].

Nucleic acid amplification by PCR was first applied for M.pneumoniae diagnosis in 1989 by Bernet et al. the main advantage of PCR is its superior sensitivity. The sensitivity may be increased by nested PCR which may be required for detection of M.pneumoniae from extrapulmonary sites. Multiplex PCR assays were designed for simultaneous detection of M. pneumoniae and other respiratory pathogens. RNA amplification techniques are used for their high sensitivity and detection of this nucleic acid is more indicative of viable mycoplasmas in clinical samples.

Antimicrobial susceptibilities and chemotherapy

Infections caused by M.pneumoniae are generally treated with tetracycline or erythromycin. Because of the difficulty in culturing the organism, its slow growth rate and the lack of a readily available method, antimicrobial susceptibility testing of M.pneumoniae is neither necessary nor appropriate. Antimicrobial susceptibility testing of M.pneumoniae strains indicate that this organism is susceptible to a wide variety of antimicrobial agents including the quinolones that is cipfloxacin , levofloxacin, ofloxacin, gemifloxacin, moxifloxacin, clindamycin, lincomycin, tetracycline, minocycline, doxycycline, erythromycin, streptomycin. Some of the newer macrolide antibiotics such as clarithromycin, azithromycin, flurithromycin are also highly active against M.pneumoniae and show a very narrow range of minimal inhibitory concentrations.

MATERIALS AND METHODS

This prospective study was conducted on 100 HIV sero-positive patients attending the outpatient department or admitted in the Medical, Venereology and Thoracic Medicine Departments with signs of lower respiratory infection at Coimbatore Medical College Hospital, Coimbatore. Specimens for the study were collected over a period of one year and eight months from January 2005 to August 2006 .

Approval was obtained from the ethical committee prior to conducting the study and Informed consent from all patients under study was also obtained.

- Inclusion criteria:

  One hundred consecutive HIV sero-positive patients with symptoms and signs suggestive of lower respiratory tract infection and who have not received antibiotics for minimum of 2 weeks prior to this study.

- Exclusion criteria:

  Pediatric HIV sero-positive patients were excluded.

The name, age, sex, address, date of admission, inpatient number, clinical history of the patient were noted. A thorough general examination and systemic examination of the patient was also done.

HIV antibodies had been determined by Enzyme immuno assay by micro ELISA and confirmed by rapid ELISA (Immuno chromatography and Immuno comb).

HIV seropositive patients were classified under different clinical stages[95] according to the world health organization [WHO] guidelines as mentioned in appendix i.

Basic investigations such as Hb%, TC, DC, LFT, RFT, Blood Sugar, Urine complete examination, chest x-ray were also done.

CD4 cell counts assays done by flow-cytometry and immune status grading of the patients was done according to the WHO [95] guidelines as mentioned in appendix ii.

Sputum specimens were collected for bacterial, fungal and mycobacterial cultures.

Blood cultures were done for all patients to find out any bacteraemia.

Specimen collection and transport:

Samples collected: Expectorated sputum

Induced sputum

Blood

Collection of expectorated sputum sample:

The patient was instructed to cough out deeply and early morning sputum samples on three consecutive days were collected in sterile wide mouthed containers fitted with screw capped lids.

Collection of induced sputum sample:

Induction of sputum was done using a nebuliser with 3% hypertonic saline for 15 minutes and sputum was collected in sterile wide mouthed containers.

Collection of blood sample:

After cleansing the site for venepuncture with betadine and 70% alcohol about 7 ml of blood was collected and 5 ml was added to 50 ml of sterile brain heart infusion broth in blood culture bottles and remaining 2 ml of blood was used for serological tests.

All the specimens were sent to the laboratory immediately after collection.

Safety precautions

- All sputum samples were potentially infectious and leak proof containers were used for collection and transportation of the samples.
- Biological safety cabinet level III was used for carrying out all procedures involving sputum and protective wears like mask, gloves etc was used.
- Disinfection of the sputum cups/ containers by treating with freshly prepared 1-2% sodium hypochlorite solution or autoclaving was followed.

Processing of sputum samples:

Induced sputum was used for detection of trophozoites and cysts of P. jiroveci.

Expectorated sputum samples were used for isolation of bacterial, mycobacterial and other fungal pathogens.

The quality of expectorated sputum was assessed by macroscopic examination and microscopic examination. Any sample that was thin, watery and with no purulent matter was considered unsuitable for further processing. Gram stain was done and Brahmadhatan et al[94] system was used for assessing the quality of sputum samples as described in appendix iii.

The following staining methods were carried out for detection of various organisms as described in the table below:

TABLE 2 – Stains used in our study

Methods	Organisms
Gram stain	For assessing quality of sputum & bacteria.
Ziehl Neelson stain (25% acid fast)	Mycobacteria
Ziehl Neelson stain (1% acid fast)	Nocardia
10% KOH mount	Fungi
Indian ink preparation	Encapsulated yeasts
Giemsa stain	Fungi, P.jiroveci (trophozoites)
Toluidine blue O stain	P. jiroveci (cysts)

Preparation and procedure for staining

Gram's stain Procedure

1. Appropriate smear is made on a clean glass slide.

The smear is fixed by passing the slide over flame 2-3 times quickly.

Cover the slide with crystal violet solution and allow to act for about 30 seconds.

2. Pour off stain and holding the slide at an angle downwards pour on the iodine solution on the slide so that it washes away the crystal violet. Cover the slide with fresh iodine solution and allow to act for 1 minute.

3. Decolorize with 100% acetone. First tip off the iodine and hold the slide at a steep slope. Then pour acetone over the slide from its upper end, so

as to cover its whole surface. Decolorization is very rapid and is usually complete in 2-3 seconds. After this period of contact, wash thoroughly with water under a running tap

4.Apply the counterstain (0.5% safranine) for 30 seconds.

5. Wash with water and blot dry.

6. Examine the smear under oil immersion microscopy.

Ziehl Neelsen Staining Procedure

Reagents

– Carbol fuchsin ---- 1%

– Sulphuric acid ---- 25%

– Methylene blue --- 0.1%

Method

1. Select a new, unscratched slide and label the slide with a laboratory serial number.
2. Make a smear from yellow purulent portion of the sputum using the jagged end side of a bamboo stick. A good smear is spread evenly, 2cms x 3cms in size and is neither too thick nor too thin. The optimum thickness of the smear can be assessed by placing the smear on a printed matter, the print should be just readable through the smear.
3. Let the smear air-dry for 15-30 mins.
4. Fix the smear by passing the slide over the flame 3-5 times for 3-4 seconds

each time.

5. Place the fixed slide on the staining rack with the smeared side facing upwards.

6. Pour filtered 1% carbol fuchsin over the slide so as to cover the entire slide. Do not leave the carbol fuchsin on the slide for a long time (not more than 5 mins.)

7. Heat the slide underneath until vapours start rising. Do not let carbol fuchsin to boil or the slide to dry. Continue the process up to five minutes.

8. Allow the slide to cool for 5-7 minutes.

9. Gently rinse the slide with tap water to remove the excess carbol fuchsin stain. At this point, the smear on the slide looks red in colour.

10. Decolour the stained slide by pouring 25% sulphuric acid on the slide and leaving the acid for 2-4 mins.

11. Lightly wash away the free stain. Tip the slide to drain off the water. If the slide is still red, reapply sulphuric acid for 1-3 mins, and rinse gently with tap water.

12. Counter stain the slide by pouring 0.1% methylene blue solution on to slide and let it stand for one min.

13. Gently rinse the slide with the tap water and tip the slide to drain off the water.

14. Place the slide in the slide tray and allow it to dry.

15. Examine the slide under the microscope using 40 x objectives to select the suitable area of the slide and examine under the 100 x lenses using

a drop of immersion oil for the characteristic acid fast bacilli. At least 100 oil immersion fields should be examined before declaring a smear as negative. In case of the scanty result, examine another 100 oil immersion fields.

TABLE 3 - Grading of sputum smears for AFB was done as follows :

Examination findings	Result	Grading	Minimum No of fields to be examined
More than 10 AFB per oil immersion	Positive	3+	20
1-10 AFB per oil immersion	Positive	2+	50
10-99 AFB per 100 oil immersion	Positive	1+	100
1-9 AFB per 100 oil immersion	Scanty	Record exact number seen	200
No AFB per 100 oil immersion fields	Negative	0	100

A portion of the induced sputum specimen was washed with saline and centrifuged at 1,500 rpm/10 minutes and the sediment was smeared and stained with Giemsa and Toluidine blue O and examined for cysts and trophozoites of P. jiroveci.

Giemsa stain

Preparation and procedure for staining[94]

Preparation:

Giemsa powder 0.3 gm

Glycerine 25.0 ml

Acetone free methanol 25.0 ml

The stock solution is diluted before use by adding 1 ml of stain to 10 ml of distilled water.

Procedure for staining:

1. Air dry thin films.
2. Fix in methanol for 1 minute
3. Wash in tap water and flood the slide with Giemsa diluted 1in 10 with buffered distilled water (pH 7.2). The diluted stain must be freshly prepared each time.
4. Stain for 25 – 30 minutes.
5. Run tap water on to the slide to float off the stain and to prevent deposition of precipitate on the film.
6. Examine the film using X 100 objective.

Toluidine blue O stain

Preparation and procedure for staining[94]

Preparation:

Toluidine Blue 0.3 gm

Concentrated HCl 2.0 ml

Absolute alcohol 40.0 ml

Distilled water 60.0 ml

Dissolve the stain in absolute alcohol. Add 60 ml water and finally add 2 ml of conc. HCl.

Procedure for staining

1. Fix the smear in sulfation reagent (4.5 ml glacial acetic acid + 1.5 ml concentrated sulfurinc acid) for 10 minutes. Mix the reagent with a glass rod immediately and 5 minutes after.
2. Wash in a gentle stream of running tap water for 5 minutes.
3. Dip the slide in Toludine blue O stain, for 10 minutes.
4. Decolourise in 95% alcohol / absolute alcohol (by taking in and out) 5 secongs X 2.
5. Dip in xylene for cleaning – 10 seconds.
6. Mount with mounting fluid.
7. Observe with X 20 and X 40 objectives.

The cyst wall stains violet to purple. Thickenings and folds in cyst wall stain darker violet to purple. Trophozoites and intracystic bodies are not stained.

Culture of sputum specimen

The sputum samples were inoculated onto blood agar with 10% sheep blood, chocolate agar with 10% sheep blood, Mac Conkey agar. Inoculated blood agar and chocolate agar plates were incubated at 37°C with 5-10% CO_2 and Mac Conkey agar was incubated at 37°C.

The sputum was inoculated onto Sabouraud's Dextrose agar(SDA) with antibiotics and SDA without antibiotics in duplicate (incubated at 37°C and 25°C).

A portion of sputum specimen was decontaminated as per modified Petroff's method as described[94] and inoculated in Lowenstein Jensen's (LJ) medium for culturing mycobacteria. Inoculated LJ medium bottles were incubated at 37°C for 6-8 weeks and observed for any growth.

All the bacterial and fungal pathogens isolated were identified as per standard protocol[92]. Antimicrobial susceptibility test of the bacterial isolates to various antibiotics was also done by Kirby Bauer's disc diffusion method and antibiotic sensitivity pattern studied according to clinical laboratory standards institute(CLSI).

Processing of sputum for culture of M. pneumoniae:

Homogenisation of expectorated sputum specimen was done by repeatedly drawing through a needle and syringe as chemical treatments for sputum liquefaction are toxic for mycoplasma.

Isolation and identification of M. pneumoniae was performed as described by Koneman et al[92].

Inoculation and Isolation: About 0.2 ml of homogenized sputum specimen was inoculated in a tube of Mycoplasma selective diphasic culture medium. The test tube was then loosely sealed and incubated at 35°C for upto 4 weeks and inspected daily for turbidity and colour change. (Broth phase – Violet to green or yellowish green. Agar phase – purple to yellow or yellow orange). If there was a

change in the colour of the medium with no increase in turbidity several drops of broth culture were subcultured onto a mycoplasma selective agar medium plate and was incubated at 35°C with 5% CO_2 for another 7 days. The agar surface observed for colonies after the fifth day under 10 X and 40 X magnification had the appearance of fried eggs.

Blind subcultures of broth to agar medium was done in the absence of obvious colour change after 1 and 3 weeks of incubation.

Commercially available PPLO broth and agar base from Hi Media was used and PPLO diphasic medium and agar was prepared for selective isolation of M. pneumoniae and selective supplements added as follows:

Procedure for preparation of methylene blue glucose diphasic medium and agar PPLO diphasic medium and agar.

(I) Base reagents for preparation of complete media

A. Mycoplasma base agar: This medium may be prepared using commercial PPLO agar base according to the package instruction.Basal ingredients in PPLO agar base are as follows:

Beef heart infusion	250 gm
Peptone	10 gm
NaCl	5 gm
Purified agar	14 gm
Distilled water	1 ltr

Mix together and melt the agar in a boiling water bath. Dispense in 70 ml aliquots and autoclave at 15 lbs for 15 minutes. Store at 4°C.

B. Yeast extract:

1. Purchase commercial 25% yeast extract or prepare as follows – weigh out 250 gm of active baker's yeast and place in 1 ltr of distilled water. Heat to boiling, cool and filter to remove particular matter. Adjust the pH to 8.0 and filter – sterilize.

2. Yeast extract (20ml) is then mixed with uninactivated horse serum (10ml) in a 2:1 ratio and frozen in 30 ml aliquots at -20°C.

C. Phenol red solution (0.4%): Phenol Red (1 g) is dissolved in 3 ml of 1 N NaOH and then 247 ml of distilled water is added. Solution is filtered – sterilized and stored at 4 to 8°C.

D. Methylene Blue solution (1%): Methylene blue (1gm) is dissolved in 100 ml distilled water and autoclaved for 15 minutes.

E. Thallium acetate solution(10%): Thallium acetate (10g) is dissolved in 100 ml of distilled water and filter sterilized.

F. Glucose solution: Glucose (50 gm) is dissolved in 100 ml distilled water and filter sterilized.

G. Penicillin solution: Penicillin powder is dissolved in distilled water to achieve a concentration of 1,00,000 units / ml.

(II) Methylene Blue – Glucose Diphasic Medium:

1. Melt 70 ml of mycoplasma agar in a boiling water bath. Cool to 50°C and add 30 ml prewarmed yeast extract serum.

2. Add the following:

Methylene blue solution (1%)	0.1 ml
Phenol red solution (0.4%)	0.5 ml
Glucose solution	2.0 ml
Thallium acetate solution (10%)	0.25 ml
Penicillin solution	3.0 ml

3. Adjust the pH to 7.8 with sterile 1 N NaOH.

4. Dispense agar in 1 ml aliquots into sterile 13 X 100 mm screw capped tubes.

5. Mix 140 ml of mycoplasma broth (A, above, without agar) with 60 ml of yeast extract serum. Add the following to this mixture

Methylene blue solution (1%)	0.2 ml
Phenol red solution	1.0 ml
Glucose solution	4.0 ml
Thallium acetate solution (10%)	6.0 ml
Pencillin solution	0.5ml

6. Adjust the pH to 7.8 with sterile 1 N NaOH. Dispense 2.0 ml into each of the tubes containing the solidified agar medium.

(III) Mycoplasma glucose agar medium

1. Melt 70 ml of mycoplasma agar in a boiling water bath. Cool to 50°C.

2. Prewarm 30 ml of yeast extract serum and mix with the agar medium

3. Aseptically add the following reagents:

Glucose solution	2.0 ml

Thallium acetate solution (10%)	0.25 ml
Penicillin solution	3.0 ml

4. Adjust the pH to 7.8 with sterile 1 N NaOH.

5. Dispense 6 ml of medium into 60 X 15 mm Petri dishes. Allow to cool. Store media at 4°C.

Identification: Presumptive identification of colonies of mycoplasma pneumoniae was done by the following methods:

1.Diene's stain, 2.Haemadsorption, 3.Glucose fermentation, 4.Hydrolysis of urea, 5.Arginine hydrolysis.

Diene's stain procedure for identification of mycoplasmas:

I. Principle: Mycoplasma colonies from are easily identified by observing typical colonies on agar medium. Visualization of colony morphology is facilitated by application of Diene's stain directly to agar surface.

Diene's stain is a nonspecific stain that imparts a contrasting appearance of mycoplasma colonies on agar, allowing easier visualization of colony morphology and characteristics.

II. Reagents: A. Modified Diene's stain (stock solution)

Methylene blue	2.5 gms
Azure blue	1.2 gm
Maltose	10.0 gm
Na_2CO_3	0.25 gm
Distilled water	100.0 ml

Diene's stain working solution is prepared by diluting an aliquot of the stock solution 1:3 with distilled water.

(III) Procedure:

1. Flood an agar plate containing mycoplasma growth with 1 ml of Diene's stain working solution.
2. Immediately rinse the agar surface with distilled water to remove the stain.
3. Decolorize the medium by adding 1 ml of 95% ethanol. Leave in contact with the agar for 1 minute, then remove. Repeat the wash step a second time.
4. Rinse the plate with distilled water and allow to dry.
5. Observe for colonies under the low power of a microscope (50 to100X).

IV Results: A. Interpretation:

Mycoplasmas with the 'fried – egg' colony morphology will stain with a dark blue centre and light blue periphery and will appear highly granular. The agar background will be clear or slightly violet.

Mycoplasma other than M. pneumoniae will remain stained; M. pneumoniae reduces the methylene blue after a time and will become colorless.

Hemadsorption test for identification of mycoplasma pneumoniae

(I) Principle: Among the respiratory Mycoplasmas, M. pneumoniae is the only species that will specifically absorb red blood cells. This property,therefore, provides a method for the presumptive identification of M. pneumoniae.

When colony growth is noted on mycoplasma isolation media inoculated with respiratory tract specimens a suspension of guinea pig erythrocytes is placed on the agar surface for a given time and then washed off. M. pneumoniae colonies will adsorb some of the red cells to the colony surface.

(II) Reagents:

A. Mycoplasma glucose agar with suspicious colonies present
B. Washed guinea pig erythrocytes (0.2 – 0.4%) suspended in mycoplasma broth medium.

(III) Procedure:

1. Flood the surface of the agar plate with 2 ml of red cell suspension.
2. Incubate the plate at 35°C for 30 minutes and rotate the plate occasionally to prevent the red cells from settling out.
3. Wash the surface of the plate three time with 3 ml of mycoplasma broth by gently rotating the plate. Remove wash fluid by aspiration with a pipet.
4. Examine the colonies at 50 to 100X magnification under a dissecting microscope.

(IV) Results:

A. Interpretation:

1. Positive test: Colonies with red cells adsorbed onto the surface – M. pneumoniae

2. Negative test: Colonies with no red cells adsorbed – Mycoplasma species, not M. pneumoniae.

Glucose fermentation:

Sterile PPLO broth with 0.5% glucose with pH 7.8 prepared as described above and about 2 ml of medium was dispersed in 13 X100 mm sterile screen capped tubes.

0.25 ml of positive broth culture was inoculated and incubated aerobically at 37°C for 10 days. Fermentation status was checked daily. A change in colour from purple to yellow was noted.

Arginine hydrolysis

Sterile screw capped tubes containing 2 ml of Arginine broth with 0.25% arginine at pH 7.0 was inoculated with 0.25 ml of broth culture of the isolate and incubated aerobically at 37°C for 10 days. The result of hydrolysis was checked daily by observing a colour change from purple to red.

Urea hydrolysis

Ten percent Urea was incorporated into PPLO broth media at a final concentration of 1% in the media and pH of the medium was adjusted to 6.5. Screw capped tubes with sterile medium was inoculated with 0.25 ml of broth

culture of the isolate and incubated aerobically at 35°C for 10 days. Urea breakdown was checked based on observing colour change from purple to red.

Serological tests

About 2 ml of blood was centrifuged and serum was separated and commercially available Mycoplasma pneumoniae IgM TMB ELISA (Bio Rad Laboratories) was done according to manufacturer's instructions supplied in the kit.

Principle : When antigens bound to the solid phase are brought into contact with a patient's serum, antigen specific antibody, if present, will bind to the antigen on the solid phase forming antigen-antibody complexes. Excess antibody is removed by washing. This is followed by the addition of goat anti-human IgM globulin conjugated with horseradish peroxidase, which then binds to the antibody-antigen complexes. The excess conjugate is removed by washing, followed by the addition of substrate and chromogen, Tetramethylbenzidine (TMB). If specific antibody to the antigen is present in the patient's serum, a blue color develops. When the enzymatic reaction is stopped with 1N H2SO4, the contents of the wells turn yellow. The color, which is proportional to the concentration of antibody in the serum, is read on ELISA microwell plate reader at 450nm.Serological response of the patients against mycoplasma antigen coated to microtitre wells is thus determined.

Serum diluent used contained goat antihuman IgG to adsorb and remove competing IgG.Cut off calibrators were used to calibrate the assay to account for

day to day fluctuations in temperature.Horse radish peroxidase conjugate containing goat antihuman IgM and tetra methyl benzidine substrate were used.

Assay Procedure

1. Place the desired number of strips into a microwell frame. Allow six (6) Control/Cutoff Calibrator determinations (one Negative Control, three Cutoff Calibrators, one High Positive Control, and one Low Positive Control) per run. A reagent blank (RB) should be run on each assay.
2. Dilute test sera, Cutoff Calibrator and Control sera 1:81 (e.g., 10 µL + 800 µL) in Serum Diluent Plus
3. To individual wells add 100 µL of diluted patient sera, Cutoff Calibrator and Control sera. Add 100 µL of Serum Diluent Plus to the reagent blank well.
4. Incubate each well at room temperature (21° to 25° C) for 30 minutes + 2 minutes.
5. Using automated washing equipment add 250-300 µL of diluted Wash Buffer to each well. Repeat the wash procedure four times (for a total of five washes) for automated equipment
6. Add 100 µL Conjugate to each well, including the reagent blank well.
7. Incubate each well 30 minutes + 2 minutes at room temperature (21° to 25° C).
8. Repeat Wash as described in Step 5.

9. Add 100 µL Chromogen/Substrate solution (TMB) solution to each well, including reagent blank well10. Incubate each well 10 minutes + 2 minutes at room temperature (21° to 25° C).

11. Stop reaction by addition of 100 µL of Stop Solution (1N H2SO4) following the same order as Chromogen/Substate addition, including reagent blank well. Tap the plate gently along the outsides to mix contents of the wells. The plate may be held up to one hour after addition of the Stop Solution before reading.

12. The developed color should be read on an ELISA plate reader equipped with a 450 nm filter.

Calculations

1. Mean Cutoff Calibrator O.D. (Optical Density) - Calculate the mean O.D. value for the Cutoff Calibrator from the three Calibrator determinations.

2. Correction Factor - To account for day-to-day fluctuations in assay activity due to room temperature and timing, a Correction Factor is determined for each lot of kits. The Correction Factor is printed on the Calibrator vial.

3. Cutoff Calibrator Value - The Cutoff Calibrator Value for each assay is determined by multiplying the Correction Factor by the mean Cutoff Calibrator O.D.

4. ISR Value - Immune Status Ratio (ISR) for each specimen is calculated by dividing the specimen O.D. Value by the Cutoff Calibrator Value

Immune Status Ratio (ISR) was calculated as per manufacturer's instructions.

TABLE 4 -(ISR) Immune Status Ratio Interpretation:

ISRValue	Results	Interpretation
</= 0.9	Negative	No detectable IgM antibody to Mp suggests no current immunological exposure to Mycoplasma pneumoniae.
0.9 – 1.09	Equivocal	Immunological exposure cannot be assessed. Samples should be retested by an alternate method (IFA). If results remain equivocal upon further testing an additional sample should be taken.
>/= 1.10	Positive	Significant level of detectable IgM antibody to mycoplasma. Indicative of current or recent infection.

RESULTS

This study included 100 HIV seropositive patients with lower respiratory tract infections.

TABLE 5 - AGE & SEX-WISE DISTRIBUTION OF CASES

Sex	No. of patients	Age in years			
		Range	Mean	Median	Percentage
Male	71	27 – 55	37	38	71
Female	29	28 – 65	36	35	29

Among the 100 cases, 71 were males with range of age from 27 – 55 years and mean age 37.6 and 29 were females with range of age from 28 – 65 years and mean age of 36.2.

TABLE 6 - AGE GROUPS UNDER STUDY

	Age in years				
	21 – 30	31 – 40	41 – 50	51 – 60	> 60
Male	14	35	20	2	-
Female	8	17	3	-	1
Total	22	52	23	2	1

Among the 71 males,14 were in 21 -30 year age group, 35 were in 31 – 40 year age group, 2 were in 51-60 year age group and among the females, 8 were in 21 – 30 year age group, 17 were in 31 -40 year age group and 2 were in 40 – 50 year and 1 female was 65 years of age.

TABLE 7 - PATHOGENS ISOLATED FROM HIV SEROPOSITIVE PATIENTS

Pathogens isolated	No. of HIV infected patients N=100
Mycobacteria	21
Mycoplasma pneumoniae	19
Other bacteria	54
Fungi	19
Polymicrobial	29
Total no. of culture positive cases	70
Total no. of pathogens isolated	113

Among the 100 cases included in the study, pathogens could be isolated from 70 patients. Polymicrobial isolation was found in 29 cases.

TABLE 8 - AGE & SEXWISE DISTRIBUTION OF CULTURE POSITIVE CASES IS AS FOLLOWS

	21 – 30	31 – 40	41 -50	51 – 60	> 60
Males	12	24	12	2	-
Females	6	11	2	-	1
Total	18	35	14	2	1

Etiological agent could be identified in more number of patients in 21 – 30 and 31 – 40 year age groups.

Among the study cases most common clinical presentations were cough(95%), dyspnea(80%) , chest pain(82%) , fever(76%) , night sweats(73%) , Loss of appetite(70%) , haemoptysis , neutropenia(56%) , anaemia(26%) and thrombocytopenia(32%) and 46% of cases were on antiretroviral therapy.

TABLE 9 - BACTERIAL PATHOGENS ISOLATED FROM HIV SERO-POSITIVE PATIENTS

Pathogens isolated	Number of isolates N=73	In single / Mono-microbial	In combination / Polymicrobial
Klebsiella pneumoniae	22	12 (54.5%)	10 (45.4%)
Mycoplasma pneumoniae	19	4 (21%)	15 (79%)
Pseudomonas aeruginosa	10	5(50%)	5 (50%)
Staphylococcus aureus	9	3 (33%)	6 (67%)
Streptococcus pneumoniae	8	5 (62%)	3 (38%)
Moraxella catarrhalis	3	1 (33%)	2 (67%)
Haemophilus influenzae	1	1	-
Escherichia coli	1	-	1

The most common bacteria isolated were Klebsiella pneumoniae (n=22), Pseudomonas aeruginosa (n=10), Staphylococcus aureus (n=9), Streptococcus pneumoniae (n=8) which constituted 30%, 13%, 12%, 11% of the total bacterial isolates respectively and the numbers they were isolated in combination with other organisms were as shown in the table.

TABLE 10 - MYCOBACTERIA ISOLATED FROM HIV SERO-POSITIVE PATIENTS

Mycobacteria isolated	No. of cases	In single	In combina-tion
Mycobacterium tuberculosis	20	4	16
Atypical mycobacteria	1	-	1

21 Mycobacteria were isolated from our study. Most of the organisms (16) were polymicrobial isolations. One atypical mycobacterium was isolated from a patient with stage III disease and CD4 count of 115 cells/mm^3. The atypical mycobacterium was presumptively identified as Mycobacterium avium intracellulare.

TABLE 11 - FUNGAL PATHOGENS ISOLATED FROM HIV SERO-POSITIVE PATIENTS

Pathogens isolated	No. of cases	In single / Monomicrobial	In combination / Polymicrobial
Candida species	15	-	15
Aspergillus niger	2	1	1
Cryptococcus neoformans	1	-	1
Penicillium marneffei	1	-	1

19 pathogenic fungi were isolated in our study, out of which, 15 were Candida species. Gram stained smears of patients showing pseudohyphae and yeast cells were only considered as pathogenic isolates. Sputum examination was done for 3 consecutive days to confirm the fungal isolates.

Cryptococcus neoformans was isolated in a case diagnosed to have pulmonary tuberculosis on anti tuberculous treatment and that patient succumbed to death. This patient had CD4 cell count of 120 cells / mm^3.

Penicillium marneffei was also isolated in a patient with CD4 cell count of 125 cells/mm^3.

Aspergillus niger was isolated in two cases and both cases had CD 4 counts < 200 cells /mm^3. This was confirmed by repeated sputum culture on 3 consecutive days.

TABLE 12 - IMMUNOSTAGING IN STUDY CASES AND SPUTUM CULTURE POSITIVE CASES

Levels of Immuno-suppression	CD4 count Cells/ mm^3	Total No. of cases N=100	No. of culture positive cases N=70
Severe IS	<200	43	38
Advanced IS	200 – 349	52	29
Mild IS	350 – 499	5	3
No significant IS	> 500	0	0

TABLE 13 - HIV CLINICAL STAGING IN STUDY CASES AND SPUTUM CULTURE POSITIVE CASES

HIV stage	Total No. of cases N=100	No. of culture positive cases N=70
I	2	0
II	43	30
III	51	36
IV	4	4

Revised WHO clinical staging and immunological staging for classification of HIV seropositive cases was used in our study.According to this 67(97%) of

culture positive cases were in stages of advanced and severe immunosuppression with CD4 counts of 200-349 and <200 cells /mm^3 respectively.

30 sputum culture positive cases were in clinigal stage II and 36 cases were in clinical stage III and 4 cases were in stage IV and this constituted a total of 94% in clinical stages II and III.

TABLE 14 - AGE AND SEX-WISE DISTRIBUTION OF CULTURE POSITIVE CASES OF MYCOPLASMA PNEUMONIAE IN HIV SERO-POSITIVE PATIENTS

Age group in years	No. of males N=71	Culture +ive for Mycoplasma pneumoniae		No. of females N=29	Culture +ive for Mycoplasma pneumoniae	
		No. N=14	%		No. N=5	%
21 – 30	14	3	21.4	8	1	12.5
31 – 40	35	7	20	17	3	17.6
41 – 50	20	4	20	3	1	33.3
>50	2	-	-	1	-	-

Mycoplasma pneumoniae was isolated in 19 cases, out of which 14 were males and 5 were females.Mycoplasma isolation was found to be more in n=10 in 31-40 year age group than in other age groups.M.salivarium was also isolated in 2 cases but it was excluded as a commensal.

TABLE 15 – CO-PATHOGENS ISOLATED ALONG WITH MYCOPLASMA PNEUMONIAE

Pathogens isolated	Mycoplasma pneumoniae positive cases N = 19	Mycoplasma pneumoniae negative cases N = 81
Klebsiella pneumoniae	5 (26%)	17 (20.9%)
Pseudomonas aeruginosa	3 (16%)	7 (8.6%)
Staphylococcus aureus	3 (16%)	6 (7.4%)
Moraxella catarrhalis	0 (0%)	3 (3.7%)
Streptococcus pneumoniae	1 (5%)	7 (8.6%)
Mycobacteria	6 (32%)	15 (18.5%)
Aspergillus niger	1 (5%)	1 (1.2%)
Candida species	5 (26%)	10 (12.3%)

Klebsiella pneumoniae (n=5),Pseudomonas aeruginosa(n =3),Staphylococcus aureus(n =3),Mycobacteria(n=6) were the most common co-pathogens isolated along with Mycoplasma pneumoniae positive cases in combination.

TABLE 16 - ANTIMICROBIAL SUSCEPTIBILITY PATTERN OF BACTERIAL ISOLATES

Pathogens	Gentamicin 10µg	Amikacin 30µg	Ciprofloxacin 5µg	Ofloxacin 5µg	Cefotaxime 30µg	Ceftrioxazone 30µg	Cotrimoxazole 25µg	oxacillin 1µg	Amoxicillin 20µg	Vancomycin 30µg	Erythmycin 15µg
Klebsiella pneumonia (N=22)	10	14	14	15	14	15	5	-	-	-	-
Pseudomonas aeroginosa (N=10)	4	5	6	7	4	5	3	-	-	-	-
Haemophillus influenzae (N=1)	1	1	1	1	1	1	1	-	-	-	-
Escherichia coli (N=1)	1	1	1	1	1	1	0	-	-	-	-
Staphylococcus aureus (N=9)	-	5	5	6	6	8	4	5	4	9	4
Streptococcus pneumoniae (N=8)	-	7	5	6	7	8	6	7	7	8	7
Moraxella catarrhalis (N=3)	-	2	2	2	2	2	1	2	1	2	1

The above values mentioned in the table are the number of isolates found to be sensitive to the corresponding antibiotics as above.

Klebsiella pneumoniae was 63% and 69% sensitive to fluoroquinolones and third generation cephalosporins respectively and 30% were extended spectrum betalactamase producing strains.

Pseudomonas aeruginosa was 70% sensitive to fluoroquinolones. Staphylococcus aureus was 89% sensitive to third generation cephalosporins and 24% were methicillin resistant strains.

Streptococcus pneumoniae was 87% sensitive to fluoroquinolones, erythromycin and cephalosporins.

Antibiotic Sensitivity pattern of other bacterial isolates are as mentioned in the table above.

TABLE 17 - CD4 CELL COUNT IN CULTURE

POSITIVE CASES OF MYCOPLASMA PNEUMONIAE

HIV STAGING IN CULTURE POSITIVE CASES OF MYCOPLASMA PNEUMONIAE

HIV stage	No. of culture positive cases N=19
I	0 (0%)
II	4 (21%)
III	11 (57.8%)
IV	4 (21%)

12(63%) cases with sputum culture positive for M.pneumoniae had CD4 counts <100 cells /mm^3 and all these cases had mixed infections with other organisms and 11(57.8%) , 4(21%) of mycoplasma positive cases had stage III and stage IV disease respectively.

TABLE 18 - MYCOPLASMA PNEUMONIAE IgM ELISA POSITIVE CASES WITH CULTURE POSITIVE CASES

Mp IgM ELISA positive cases (N)	Mp IgM ELISA positive and Mp culture positive cases(N)	MpIgM ELISA positive and Mp culture negative cases(N)
23	19	4

The incidence of mycoplasma pneumoniae was 19% in our study group. Mycoplasma pneumoniae IgM ELISA showed positivity in 23% of cases among which sputum culture for mycoplasma pneumoniae was positive in 19%.

Blood cultures were found to be positive (n=16) in our study cases along with sputum culture positivity. The most common isolate was Klebsiella pneumoniae(N=8) followed by Pseudomonas aeruginosa(N=3), Staphylococcus aureus(N=5).

Antibiotic sensitivity pattern of the isolates was similar to the isolates from sputum culture positivity.

TABLE 19 - CLINICORADIOLOGICAL FINDINGS OF PATHOGENIC ISOLATES AMONG HIV SERO-POSITIVE PATIENTS

Clinical Diagnosis	Radiology	Isolates
Broncho pneumonia N = 10	Peribronchial infiltrates	Staphylococcus aureus Mycoplasma pneumoniae Moraxella catarhalis Aspergillus niger
	Interstitial infiltrates	Haemophillus influenzae
Lobar pneumonia N = 9	Consolidation	Klebsiella pneumoniae Streptococcus pneumoniae
	With cavitations	Pseudomonas aeroginosa
	With pneumothorax	Strepococcus pneumoniae Mycoplasma pneumoniae
	With pleural effusion	Staphylococcus aureus
Pulmonary tuberculosis N = 24	Bilateral extensive cavitatory lesions	Atypical mycobacterium
	Consolidation	Mycobacterium tuberculosis
	Fibrous cavity with military mottling	Cryptococcus neoformans
	With pleural effusion	Mycobacterium tuberculosis Mycoplasma pneumoniae

Bronchitis N = 15	Increased bronchovascular marking	Klebsiella pneumoniae Pseudomonas aerogenosa
Bronchial asthma N = 12	Increased broncho-vascular markings Emphysema changes	Mycoplasma pneumoniae Streptococcus pneumoniae

Peribronchial infiltrates(10%),consolidation(9%),fibrous cavity(5%),increased bronchovascular markings(15%),emphysematous changes(10%) and pleural effusion(4%) were found in our study cases.

Staphylococcus aureus and Mycoplasma pneumoniae isolations were more common in patients with peribronchial infiltrates. Klebsiella pneumoniae,Streptococcus pneumoniae were more common isolations in patients with consolidation.Mycobacterium tuberculosis was isolated more commonly in patients with fibrous cavity and the other findings are as seen in the table.

DISCUSSION

Among the opportunistic infections associated with HIV, diseases like pneumonia of bacterial origin are encountered more frequently [10].

The importance of respiratory infections in HIV patients is well documented[3]. The true incidence of these infections is difficult to assess and varies with the population surveyed[5]. In the present study, of the 100 HIV infected patients, the etiologic agent could be identified in 70 patients.

Bacterial isolates :

In our study, the bacterial and fungal isolates from the HIV infected patients were of varied etiology and the bacterial isolates(N=54) excluding M.pneumoniae and M.tuberculosis constituted 47.7% among which K. pneumoniae isolated in 40.7%, P. aeruginosa in 18.5%, S. aureus in 16.6% and S. pneumoniae in 14.8% were the most common bacterial pathogens isolated. M. pneumoniae constituted 26% among the bacterial pathogens isolated(N=73).

As per the figures from National AIDS control organization (NACO), bacterial infections constituted 7% of opportunistic infections and the common organisms encountered in pulmonary infections were S. pneumoniae, H. influenzae, S. aureus[2].

Tchmaran[96] in his study on the lung diseases due to common bacteria in HIV infected individuals in African adults noted 81% of infections due to S. pneumoniae and reported it to be the most offending pathogen in HIV infected patients. Of all the infections reported risk or susceptibility to infections with encapsulated organisms such as S. pneumoniae and C. neoformans appear to be the most.

Falco et al [97]in their study on bacterial pneumoniae in HIV infected patients have reported S. pneumoniae in (34%), H. influenzae in 18% as the most common causative agents.

Bensel et al[98] reported S. pneumoniae and H. influenzae in 35.8% and 22.6% respectively.

Klebsiella pneumoniae , Pseudomonas aerugenosa and Staphylococcus aureus infections are usually community acquired in HIV infected patients[3].

Hirschtick et al[28] noted K. pneumoniae, P. aeruginosa in 13% and 8% of cases of confirmed pneumonia (LRTI).

Dropulic[99] in his study on the clinical manifestations of P. aeruginosa infection among patients with AIDS found that of the 73 episodes of P. aeruginosa infections, 13 were that of pneumonia.

Shailaja et al [2] in their study on LRTI in HIV infected patients have reported 9.68% of the isolates to be P. aeruginosa and also associated it as an important cause of mortality.

Moraxella catarrhalis is generally considered as a commensal in the upper respiratory tract of adults and its isolation from sputum is often considered as normal flora of the oropharaynx. This appears to be a misconception as Sehgal and Shainy[100] in 1994 reported this organisms as the second most common isolate from patients suffering from LRTI. In the present study, M. catarrhalis was repeatedly isolated from three samples collected in 3 consecutive days from 3 patients and it constituted 4% of the bacterial isolates. Gram stained sputum smears of all the these patients had numerous pus cells with many Gram negative cocci in pairs. Escherichia coli and Haemophilus influenzae constituted 4% of bacterial isolates in our study.

Mycobacterial isolates

About 55-89% of AIDS cases in India were found to be suffering from extensive pulmonary tuberculosis[4,52,53]. In our study, M. tuberculosis was isolated in 20 HIV infected patients and constituted 21.2% among the bacterial isolates and atypical mycobacterium was isolated in one the HIV infected patients and was presumptively identified as Mycobacterium avium intracellulare.

Shailaja et al [2] in their study have reported M. tuberculosis in 42.89% and atypical mycobacteria as one of the isolates. However atypical mycobacteria has been commonly reported from AIDS cases from Western countries, it has been scarcely reported from India. Shailaja et al has reported atypical mycobacteria as one of the isolates in her study.

Fungal isolates

Fungal infections occurs most often with other pathogens in immunocompromised patients [3]. In the present study, 19 fungal pathogens were isolated. In our study Candida species, Cryptococcus neoformans, Aspergillus niger, Penicillium marneffei constituted 15 (78.9%), 1 (5.2%), 2 (10.5%), 1 (5.2%), of the fungal isolates respectively. These patients had repeated isolation of the above mentioned fungi with the sputum samples collected on three consecutive days. The Gram stained smear also revealed pseudohyphae with yeast cells, and hence the possibility of esophageal colonization was ruled out.

Penicillium marneffei, a dimorphic fungus, has been most commonly reported from AIDS cases in South east Asian countries [10]. In our study, it was isolated in one of the HIV patients and it was confirmed based on yeast to mould conversion and presence of red pigment on the reverse. Cryptococcus neoformans was also isolated from one of our patients in our study.

Shanker et al [101] reported Candida species in 80% of their isolates.

Shivananda [57] in his study in 1992 on 825 patients with pulmonary infections found 15.39% of isolates to be Aspergillus species. Of these A. fumigatus was 11.15%, A. niger was 3.2% and A. flavus was 0.96%.

Geethalakshmi et al [58] has documented pulmonary aspergillosis in 36 samples.

Pankajalakshmi et al [102] emphasized the emergence of candiadiasis, cryptococcosis, aspergillosis, penicilliosis and other phacohyphomycosis in HIV infected patients.

PCP is more prevalent in Western countries.Very few Indian studies have reported P. jiroveci. Three cases of PCP have been reported from Delhi[103]. In our study, an attempt was made to demonstrate the presence of trophozoites / cysts of P. jiroveci in induced sputum. However, none of the sputum samples were positive for PCP. Elizabeth Mathai[104] has reported PC in 5 of 15 AIDS patients from broncho-alveolar lavage specimens.

Mycoplasma isolates

Though the role of mycoplasmas as cofactors in HIV has been proved, studies about the incidence of M. pneumoniae in India is scarce.In our study M. pneumoniae was isolated from 19% of the bacterial isolates.

Shankar et al [106] have reported M. pneumoniae in 36% of AIDS patients.

Dev AB et al[105]have reported 35.5% of M. pneumoniae infections in CAP. He also noted tht 60% of M. pneumoniae infections has occurred in HIV infected patients.

Louise D Teel [65] has reported mycoplasma species in 28.2% of HIV patients from BAL cultures.

Stool et al [64] have reported that mycoplasma colonized 12.5% of HIV infected patients.

Shankar et al[106] reported a higher incidence of mycoplasma pneumoniae in younger and older age group in a similar study. In our study incidence of M. pneumoniae was found to be more common in 31 – 40 year age group.

Serology:

Though direct isolation of mycoplasma is considered the gold standard, Mycoplasma culture is notoriously difficult and laborious. Hence detection of IgM antibodies against Mycoplasma pneumoniae antigen by ELISA has been done in our study as it is highly sensitive and specific and indicates recent infection. In our study, M. pneumoniae IgM ELISA was positive in 23% of

HIV infected patients. Out of these 19% of HIV infected patients had sputum culture positive for M. pneumoniae. In a study of M. pneumoniae at Yemen by Al Moyed[108], IgM antibodies against M. pneumoniae in sputum were detected in 20.7% of cases out of which 9.6% were the culture positive cases.

Phillipe tuppin et al[107] have reported M.pneumoniae IgM ELISA positivity in 15% of the cases.

Polymicrobial isolates

Polymicrobial isolation was found to be common in Mycoplasma positive HIV cases. Klebsiella pneumoniae (20.9%), Mycobacterium tuberculosis (18.5%), Staphylococcus aureus (7.4%), Pseudomonas aeruginosa (8.6%) were found to be more in mycoplasma negative cases than mycoplasma positive ones.

This was similar to the study reported by Shanker et al[106].

Clinico-radiological findings

In our study, 95% had cough as a presenting symptom followed by dyspnoea, chest pain, fever, night sweats, loss of appetite and haemoptysis. Dorigo Zetsma et al [110] in their study have reported similar symptomatology.

In our study, about 10% were clinically diagnosed to have bronchopneumonia, 9% had lobar pneumonia, 24% had pulmonary tuberculosis, 15% had bronchitis and 12% had bronchial asthma.

Almoyed et al[108] have reported 41.6% cases with bronchopneumonia, 36% cases with lobar pneumonia, 32% cases with bronchial asthma, 28% with bronchitis, 23% with tuberculosis and 20% with COPD.

In our study , 94% of sputum culture positive cases were in clinical stages II and III and about 78.9% of Mycoplasma pneumoniae culture positive cases were in III and IV stages. This was similar to study by Shankar et al[106].

Chest radiographic findings in sputum culture positive cases in our group were as follows: Peribronchial infiltrates (10%), Consolidation (9%), Fibrous cavity with military mottling (5%), Increased bronchovascular markings (15%), emphysematous changes(10%), pleural effusion (4%). Shailaja et al [2] and Robert Craven et al [111] have also reported similar findings in their group.

Peribronchial infiltrates and pleural effusion were commonly associated with Staphylococcus aureus and Mycoplasma pneumoniae infections. Consolidation was seen commonly with Klebsiella pneumoniae and Streptococcus pnuemoniae infections. Mycobacterium tuberculosis was isolated in patients with cavitatory leisions and fibrosis.

Haematological findings

In our study various hematological, immunological findings were correlated similar to the study by Shanker et al[106]. In our study, 97% of culture positive cases were in stages of advanced and severe immunosuppression with CD4 counts of 200-349 and <200 cells /mm^3 respectively. They have reported that mean CD4 counts of culture positive cases for Mycoplasma pneumoniae was 76 cells/microliter, and they had also noted that co infections were more common in Mycoplasma pneumoniae culture positive cases in AIDS cases.

In our study the mean CD4 count was 96 cells / mm^3 in Mycoplasma pneumoniae culture positive cases and 63% of culture positive cases for Mycoplasma pneumoniae had CD4 count < 100 cells/mm^3.

CD 4 cell count was less than 200 cells/mm^3 in 15% of Mycoplasma culture positive cases in a study by Phillipe Tuppin et al [107]

In our study, 56% of cases had neutropenia, 26% had anaemia and 32% had thrombocytopenia. Phillipe Tuppin et al [107] reported neutropenia, anaemia, thrombocytopenia in 48%, 20% and 28% respectively in their study group.

Antibiotic susceptibility

Klebsiella pneumoniae was 63% and 69% sensitive to fluoroquinolones and third generation cephalosporins respectively and 30% were extended spectrum betalactamase producing strains.

Pseudomonas aeruginosa was 70% sensitive to fluoroquinolones. Staphylococcus aureus was 89% sensitive to third generation cephalosporins and 24% were methicillin resistant strains.

Streptococcus pneumoniae was 87% sensitive to fluoroquinolones, erythromycin and cephalosporins.These values were similar to the study by Shailaja et al[2] and Aroma oberoi et al[109].

SUMMARY

- One hundred consecutive HIV seropositive patients with lower respiratory infection were studied during the period from January 2005 to August 2006 at Coimbatore Medical College Hospital.
- There were 71 males and 29 females in the study.
- Etiological agent was made out in 70 cases and a total of 113 pathogens were isolated from sputum culture.
- Out of the 113 pathogenic isolates bacterial isolates constituted 47.7%.
- Klebsiella pneumoniae(40.7%) was the most common bacterial pathogen isolated. This was followed by pseudomonas aeruginosa(18.5%), Staphylococcus aureus(16.6%) and Streptococcus pneumoniae(14.8%).
- Mycobacterium tuberculosis was isolated from 20% of the cases.
- Fungal pathogens isolated were Candida sp(78.9%),Cryptococcus neoformans(5.2%), Aspergillus niger(10.5%) and Penicilllium marneffei(5.2%).
- The incidence of M.pneumoniae infection was 19% in HIV seropositive patients with lower respiratory infections.Sputum culture positivity for M.pneumoniae was higher in 31-40 year age group.
- M. pneumoniae IgM ELISA positivity was found in 23% of the cases.
- 63% of culture positive cases for Mycoplasma pneumoniae had CD4 count <100 cells /mm^3
- 78.9% of Mycoplasma pneumoniae culture positive cases were in III and IV clinical stages

- 97% of culture positive cases were in stages of advanced and severe immunosuppression with CD4 counts of 200-349 and <200 cells /mm^3 respectively.94% of sputum culture positive cases were in clinical stage II and III.
- Klebsiella pneumoniae was 63% and 69% sensitive to fluoroquinolones and third generation cephalosporins respectively and 30% were extended spectrum betalactamase producing strains.
- Pseudomonas aeruginosa was 70% sensitive to fluoroquinolones.

CONCLUSION

This prospective study on one hundred HIV seropositive patients with lower respiratory infections was conducted in order to record clinical and microbiological observations to study bacterial and fungal etiology of LRTI,the incidence of M.pneumoniae infection,the significance of serology in M.pneumoniae diagnosis,the hematological and clinico radiological findings and the antibiotic susceptibility pattern of the bacterial isolates.

Klebsiella pneumoniae, Pseudomonas aeruginosa and Staphylococcus aureus were the most common bacterial isolates.M.tuberculosis was also commonly isolated.

Candida sp, Aspergillus sp, Cryptococcus neofomans, Penicillium marneffei were the fungal isolates.

We were able to isolate and identify M.pneumoniae in culture and the incidence was 19%.

M.pneumoniae IgM ELISA was positive in all the culture positive cases.Hence diagnosis of M.pneumoniae by IgM ELISA was found to be significant and hence its use has to be considered as an important diagnostic tool as the culture of M.pneumoniae is time consuming and laborious.

CD4 lymphocyte count and clinical staging of the patients and radiological and haemotological findings were also helpful in diagnosing LRTI in our study cases. As the CD4 lymphocyte count declined HIV seropositive patients were susceptible to a variety of bacterial and fungal lower respiratory pathogens.

Antibiotic sensitivity testing of the common bacterial isolates were also done.

HIV seropositive patients especially with advanced disease had increased susceptibility to lower respiratory infections.These bacterial and fungal pathogens causing lower respiratory infections were the cause of significant morbidity and mortality.

Early recognition of the onset of lower respiratory infections and identification of etiological agents associated with it are essential for treatment to succeed.The prime concern is to institute prompt antibiotic prophylactic treatment while pursuing the laboratory diagnosis.

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